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Molecular Mechanisms of Endocytosis

Meeting Review

Howard Riezman,* Philip G. Woodman,† Gerrit van Meer,‡ and Mark Marsh§
*Biozentrum of the University of Basel
CH-4056 Basel
Switzerland
†Division of Biochemistry
School of Biological Sciences
University of Manchester
Oxford Road
Manchester M13 9PT
United Kingdom
‡Academic Medical Center
University of Amsterdam
Department of Cell Biology and Histology
1105 AZ Amsterdam
The Netherlands
§MRC Laboratory of Molecular Cell Biology
University College London
Gower Street
London WC1E 6BT
United Kingdom

Cells regulate their developmental and functional programs through their interaction with the external milieu, which requires communication across the plasma membrane. The plasma membrane is constantly being remodeled by endocytosis allowing cells to control how they respond to external stimuli. Endocytosis also allows continuous sampling of the external environment, which is important for the uptake of micronutrients and for the cellular and organismal response to infectious agents. The importance of this process in human health merits its careful study and characterization. For the last nine years, a biannual European conference on endocytosis has been held in various locations. The fifth of these meetings was held September 13–18, in San Feliu de Guixols, a beautiful venue on the Costa Brava in Spain. Besides having the opportunity to scuba dive, the participants followed an interesting and ambitious schedule of oral and poster presentations on the molecular mechanisms involved in endocytosis and how these mechanisms relate to health and disease.

Vesicle Budding from the Endoplasmic Reticulum

As a basis for comparison to the seemingly more complex budding events at the plasma membrane, R. Schekman (University of California, Berkeley) presented the keynote lecture on the formation of COP-II-coated vesicles from purified, soluble components. Only three protein components are strictly required to form a COP-II-coated vesicle in vitro and these act in obligate order. Binding of Sar1p, a small GTPase, allows the Sec23 complex composed of Sec23p and Sec24p to bind. This is followed by binding of the Sec13 complex, composed of Sec13p and Sec31p (Kuehn and Schekman, 1997). Strikingly, the same components added in the correct order enabled COP-II-coated vesicles to form from protein-free phospholipid vesicles, in which acidic phospholipids are required. Therefore, the basic machinery that is needed to change membrane shape and to form a vesicle is very simple. These studies are in line with previous genetic studies that have identified a limited number of proteins that are required for COP-II-coated vesicle formation from the ER. The function of one of these, Sec12p, which promotes nucleotide exchange on Sar1p, was probably bypassed through the use of GTPγS-loaded Sar1p. Interestingly, the COP-II-coated vesicles formed from phospholipid vesicles did not have the regular shape and defined size of COP-II-coated vesicles budded from the ER.

In the endocytic pathway, it is clear that proteins are actively sorted into clathrin-coated vesicles for internalization at the plasma membrane. However, in the early secretory pathway, clear evidence for this has only recently emerged (Kirchhausen et al., 1997; Kuehn and Schekman, 1997). Schekman reported that two proteins, the general amino acid permease and pro–α factor, are actively sorted into COP-II-coated buds and that a transmembrane protein which can be crosslinked to both prepro–α factor and Sec23p might be involved in recruitment.

Vesicle Budding from the Cell Surface

In contrast to COP-II budding, budding at the plasma membrane seems to be more complicated. There are now at least 5 independent pathways for endocytic internalization; the clathrin-dependent pathway, macropinocytosis, the caveolar pathway, a clathrin- and caveolin-independent pathway, and phagocytosis. Thus far, the only common feature of these different pathways is the apparent involvement of the actin cytoskeleton. The increasing number of proteins involved in endocytosis has not only revealed the complexity of the process, but also the universality. Many of the proteins and their interacting partners are conserved from yeast to vertebrates.

A recent addition to the list of proteins involved in clathrin-mediated endocytosis is Eps15, first discovered as a substrate of the epidermal growth factor receptor kinase. Eps15 is localized to clathrin-coated pits at the cell surface, most likely through its interaction with α-adaptin. A. Dautry-Varsat (Pasteur Institute, Paris) presented evidence obtained with N. Cerf-Bensussan (INSERM, Paris) that overexpression of the COOH-terminal region of Eps15, which binds α-adaptin, inhibited clathrin-dependent endocytosis. In vitro experiments showed that this inhibition could be relieved by addition of AP-2 complex, suggesting that the inhibition was caused by sequestration of α-adaptin. A. Sorkin (University of Colorado, Denver) presented evidence that Eps15p can stimulate actin polymerization in vitro, and in collaboration with F. Brodsky (University of California, San Francisco) presented the first images of purified Eps15, a fibrous molecule with an apparent hook near one end.

The NH_2-terminal region of Eps15 has three repeated domains, termed EH domains (Eps15 homology) that have been shown to bind peptides with the sequence

NPF (Salcini et al., 1997). In yeast, two proteins with EH domains, End3p and Pan1p (Wendland et al., 1996), are required for endocytosis and affect the actin cytoskeleton. Using the two-hybrid system, S. Emr's (University of California, San Diego) group picked up proteins that interact with the EH domains from Pan1p. Among these was a yeast protein with homology to mammalian AP180, a protein that binds clathrin and localizes to clathrin-coated pits (Takei et al., 1996). There are two yeast AP180 homologs, and their NPF-rich regions interacted in vitro with Pan1p from yeast extracts. Whether the yeast AP180 proteins play a role in endocytosis is still unknown.

Many yeast proteins contain NPF motifs and two of these are clearly important for endocytosis in yeast. One is Rsp5, a ubiquitin ligase. Currently there is no evidence for a direct interaction between Rsp5 and Pan1p, but Emr reported that certain alleles of *rsp5* and *pan1* are synthetically lethal. In yeast, and most likely in animal cells as well (Riezman et al., 1996), ubiquitination of certain plasma membrane proteins is required for their internalization. H. Riezman (University of Basel) presented evidence that phosphorylation of the α-factor receptor is required for its ubiquitination and subsequent internalization. The yeast casein kinase I seems to be required for this event. R. Haguenauer-Tsapis (Institut Jacques Monod, Paris) further characterized the type of ubiquitination found on the uracil permease. This protein carries mono- to tetra-ubiquitin residues, attached to two acceptor lysines. Interestingly, these polymers are extended through Lys63 of ubiquitin. The uracil permease is the first natural target of Lys63-branched ubiquitin chains. Her data suggest that one ubiquitin per lysine is sufficient for endocytosis, although the normally ubiquitinated permease is internalized more rapidly (Galan and Haguenauer-Tsapis, 1997).

The second NPF motif-containing protein relevant to endocytosis is synaptojanin. The synaptojanins have an NH_2-terminal region with homology to Sac1, a protein involved in lipid metabolism, a central domain with inositol-5-phosphatase activity, and a COOH-terminal domain that is proline rich. P. De Camilli (Yale Medical School) presented work on yeast synaptojanin homologs. There are three genes in yeast with homology to mammalian synaptojanin. Mutations in all three genes are lethal, whereas mutations in a combination of two genes permitted growth. These mutants exhibited severe phenotypic defects, actin was delocalized and long cell surface invaginations appeared in one of the mutants, similar to those seen in the *pan1* mutant (Wendland et al., 1996). These data suggest a network of proteins connected through NPF motifs and EH domains that interact to control clathrin-mediated endocytosis and perhaps to connect it to the actin cytoskeleton. Support for this idea was presented by De Camilli. Mammalian synaptojanin (170 kDa isoform) has three NPF motifs that were shown to bind the EH domain of Eps15 in vitro. NPF motifs are also found in the AP180 homolog Calm.

Synaptojanin can also bind to amphiphysin, a protein that is a critical component for clathrin-mediated endocytosis. De Camilli and H. McMahon (MRC-Laboratory of Molecular Biology, Cambridge) presented evidence that the SH3 domain of amphiphysin binds to the proline-rich domain of dynamin and that disruption of this interaction, by microinjection of peptides or the amphiphysin SH3 domain, blocked clathrin-mediated endocytosis and recruitment of dynamin to coated pits (Shupliakov et al., 1997). McMahon presented evidence that clathrin and dynamin can compete for binding to amphiphysin. This finding could help explain how amphiphysin, which binds both dynamin and adaptins, can recruit dynamin specifically to the neck of the buds. Strikingly, dynamin, amphiphysin, AP180, and clathrin colocalize by immunofluorescence microscopy and these areas, which are likely to be the active sites of endocytosis, are associated with actin filaments.

In permeabilized cells, clathrin-coated vesicle formation involves two ATP-dependent and multiple GTP-dependent steps. E. Smythe (University of Dundee) presented a surprising result. She identified rab5-GDI as a critical cytosolic factor required for the sequestration of ligand into newly formed clathrin-coated pits. One of the other GTP-requiring steps is likely to be due to the function of dynamin. An interesting feature of dynamin that may provide insight into how it works is its potential to self-assemble into ring-like structures (presented by S. Schmid, Scripps Research Institute, San Diego). The relatively low affinity and high turnover of the dynamin GTPase suggest that it works as a mechanochemical enzyme. The GTPase activity shows cooperativity and this depends upon self-assembly that is mediated through the proline- and arginine-rich domain (PRD) of the molecule. Coassembly of wild-type and mutant (GTPase mutant) dynamins showed that the cooperativity was probably not simply due to the close proximity of GTPase domains. The pleckstrin homology (PH) domain of dynamin was a negative regulator of the assembly reaction and a region between the PRD and the PH domain stimulated GTP hydrolysis.

Another molecule that self-assembles to drive endocytosis is clathrin itself. F. Brodsky showed that clathrin hubs (the C-terminal third of the clathrin heavy chain) can bind light chain and assemble into a lattice-like network. Significantly, overexpression of hubs in cells led to a dominant-negative block in transferrin uptake but had no effect on uptake of FITC dextran nor on the distribution of the AP1 or AP2 adaptors.

The complete inhibition of clathrin-mediated endocytosis with limited effects on bulk pinocytosis using dominant-negative dynamin, hubs, and amphiphysin SH3 domains has provided further evidence for clathrin-independent pathways. Several reports on these pathways were presented at the meeting. P. Courtoy (ICP, Brussels) examined constitutive macropinocytosis in cells transformed with Src and Ras. The increase in fluid phase endocytosis due to transformation was abrogated by wortmannin and LY294002, two inhibitors of PI-3 kinase activity, and by overexpression of a dominant negative form of its regulatory subunit, p85. The increase was reproduced in nontransformed cells by overexpression of a dominant-positive p85, suggesting that PI-3 kinase induces macropinocytosis, presumably through regulation of the actin cytoskeleton.

R. Parton (University of Queensland, Australia) described the possible functions of caveolins, proteins that

can induce formation of caveolae. A novel cargo for caveolae was seen by Parton. Simian virus 40 was internalized in caveolae that were consistently smaller than the neighboring caveolae. A model was proposed in which the virus binds to the major histocompatibility class I protein on the cell surface and then recruits caveolin to form a tight-fitting membrane around the virus (Stang et al., 1997). The subsequent steps in the entry pathway are less clear but suggest that the virus reaches a domain of the rough ER before passing to the cytosol and then the nucleus.

Some protein toxins transit from the cell surface to the Golgi and/or ER to effect their entry into the cytoplasm (Sandvig and van Deurs, 1996). Ricin uptake, which can be clathrin-independent, can also be caveolin-independent because ricin is taken up by clathrin-independent endocytosis from the apical surface of MCDK cells, which is devoid of caveolin (K. Sandvig, Institute for Cancer Research, Oslo). In polarized MDCK cells, clathrin-independent endocytosis from the apical domain shows different regulation to internalization from the basolateral domain and in permeabilized cells appears to be regulated by Rho.

Recruitment into Vesicles

One of the best-characterized systems of recruitment into vesicles comes from studies of clathrin and adaptors. Adaptors interact with trafficking signals located in the cytoplasmic tails of membrane proteins. However, this does not appear sufficient to explain their interaction with membranes (Kirchhausen et al., 1997) even though increasing the amount of signal-containing proteins in the membrane increases recruitment (B. Hoflack, Pasteur Institute, Lille). One factor that affects recruitment in some cases is Arf1, which influences recruitment of AP-1 complex to Golgi membranes. M. Robinson (University of Cambridge) has explored this further by creating an Arf-dependent in vitro recruitment assay. Arf-GTP supports recruitment of AP-1 to Golgi membranes and AP-2 to late endosomes. For AP-2, but not AP-1 recruitment, phospholipase D (PLD) treatment could replace the need for Arf-GTP. Additionally, neomycin, which binds to a required cofactor of PLD, PIP2, inhibited AP-2 recruitment even in the presence of Arf-GTP, suggesting that Arf-GTP acts through PLD to stimulate AP-2 recruitment to endosomes.

A relative of the AP-1 and AP-2 complexes has been recently discovered and termed AP-3, but this complex is thought to function independently of clathrin. New evidence indicates that the AP-3 complex is required for the direct trafficking of certain lysosomal/vacuolar proteins. S. Lemmon (Case Western University, Cleveland) showed that yeast alkaline phosphatase (ALP) follows an AP-3-dependent pathway to the vacuole that is distinct from the major vacuolar protein sorting pathway. In animal cells, evidence for a similar function for AP-3 was presented by Hoflack. A lysosomal membrane protein was missorted in cells incubated with antisense oligonucleotides against the μ chain of the AP-3 complex. If one assumes that AP-3 acts like the other adaptor complexes, this suggests that AP-3 complex recognizes signals in the tails of certain lysosomal membrane proteins. Preliminary evidence presented by K. von Figura (University of Göttingen) and I. Sandoval (Autonomous University of Madrid) showed that AP-3 can interact with the LIMP II tail and that this interaction seems to depend upon a dileucine-type signal. A role for AP-3 in sorting of lysosomal enzymes is also supported by genetic evidence in D. melanogaster (Simpson et al., 1997).

K. Mostov (University of California, San Francisco) presented evidence that the small GTPase, Arf6, is localized, perhaps exclusively, to the apical plasma membrane. Overexpression of an Arf6-GTP increased the rate of apical endocytosis. Overexpression of Arf6-GDP gave a smaller increase, and overexpression of the two Arf forms together gave the largest increase. The stimulation of apical endocytosis may have been due to clathrin-dependent endocytosis because it was inhibited by overexpression of mutant dynamin although alternative explanations cannot be excluded.

Vesicle Fusion

Once a transport vesicle is formed from a donor compartment, it must be targeted to its proper destination where it can fuse with the target organelle. The importance of the Rab family of small GTPases in membrane docking/fusion has long been recognized. Rabs are active in their GTP-bound form, so that the level of membrane docking/fusion is determined by the Rab[GTP]/Rab[GDP] ratio. In this sense they can be regarded as "molecular timers" for fusion, with the length of their "on" period governed by effectors and regulators that modulate the GTPase cycle (Rybin et al., 1996). Rab5, one of the best-characterized small GTPases, is required for both fusion between coated vesicles and early endosomes and homotypic fusion between early endosomes. A downstream effector of Rab5 (Rabaptin-5), identified by the group of M. Zerial (EMBL, Heidelberg) is recruited to the membrane by Rab5-GTP and downregulates the GTPase activity of Rab5. Zerial reported the identification of a novel 60 kDa protein, Rabex-5, which forms a cytosolic complex with Rabaptin-5 and has significant sequence homology to Vps9p, a yeast protein implicated in vacuolar protein sorting. In collaboration with P. Woodman (University of Manchester), Zerial's group showed that Rabex-5 is essential for both heterotypic and homotypic fusion events on the early endocytic pathway. Rabex-5 is a potent GDP/GTP exchange factor for Rab5. Thus, the Rabaptin-5/Rabex-5 complex directly couples nucleotide exchange (and thus Rab5 activation) to effector recruitment on the membrane, thereby locally stabilizing the GTPase in the active state.

Zerial also demonstrated that Rab5-GTP is required on both membrane partners undergoing fusion. This may mean that similar Rabaptin-5/Rabex-5 complexes are recruited to both membranes. An alternative hypothesis, which might explain the intrinsic vectoriality of heterotypic fusion events, is that Rab5 could recruit distinct effector complexes on donor and acceptor membranes. Using the two-hybrid system, H. Stenmark (Norwegian Radium Hospital, Oslo) found that the early endosome-associated protein EEA1 binds to Rab5-GTP. In collaboration, Stenmark's and Zerial's groups could demonstrate binding of cytosolic EEA1 on a Rab5-GTP column,

providing independent evidence for this interaction. Evidence obtained from both in vitro and in vivo studies showed that EEA1 is essential for endosome fusion, and its association with membranes is stabilized by Rab5. Stenmark also showed that EEA1 binds specifically to PI-3P, and inhibition of PI-3 kinase causes EEA1 to translocate from endosomes to the cytosol. This may explain why Rab5-GTP prevents the inhibition of endosome fusion caused by wortmannin and other inhibitors of PI-3 kinase (Li et al., 1995), and provides additional evidence that EEA1 is a downstream effector of Rab5. Since Rabaptin-5 is mainly cytosolic at steady-state and EEA1 is associated with early endosomes, the possibility arises that EEA1 imparts vectoriality to early endosomal trafficking.

In contrast to Rabs, the NEM-sensitive fusion protein (NSF) requires nucleotide hydrolysis to promote membrane fusion (Whiteheart et al., 1994). The conventional view of NSF function is that it acts in conjunction with its cofactor α-SNAP (Soluble NSF Attachment Protein) in a late step during membrane fusion. In this model the specificity of membrane docking is determined, at least in part, by the cognate pairing of vesicle-derived (v) and target membrane (t) SNAREs (SNAP receptors) from the opposing membranes. SNAP and NSF recruitment to the bridged SNARE complex is followed by NSF-dependent ATP hydrolysis, which links docking complex disassembly directly to membrane fusion. This scenario has been challenged by several laboratories, with principal contributions from Wickner and colleagues (Mayer et al., 1996). Using a vacuole fusion assay, A. Haas (University of Würzburg) showed that Sec17p (yeast α-SNAP) and Sec18p (yeast NSF) are required in an early "priming" reaction that is completed prior to membrane docking. The priming reaction may involve the NSF and SNAP-dependent activation of the t-SNARE, Vam3p, and the v-SNARE, Nyv1p, on opposing membranes.

P. Woodman presented evidence that homotypic fusion between early endosomes also requires NSF activity at a predocking step. Woodman showed using a cross-linking approach that SNAP and NSF could be recruited to SNARE complexes on undocked membranes derived from clathrin-coated vesicles. Release of SNAP from the SNARE complex occurred upon ATP hydrolysis. These findings agree with studies of synaptic vesicle membranes (Otto et al., 1997) and are consistent with the observation of J. Heuser (Washington University, Saint Louis) that v- and t-SNAREs form parallel complexes in solution, with the components having the same orientation with respect to their transmembrane domains. Heuser showed images of NSF that provide important clues about its function during fusion. The protein hexamerizes to form a hollow cylinder, whose conformation is dependent upon nucleotide binding. In particular, a domain of NSF, which interacts with SNAP and SNAREs, adopts a more flexible conformation when ATP, versus ADP, is present. Binding of ATP to NSF would thus allow SNAP and the SNARE complex to interact with one pole of the NSF cylinder. Subsequent ATP hydrolysis would break this interaction and in some way influence SNARE conformation. How can the ability of v- and t-SNAREs to form (presumably parallel) NSF-labile complexes in the same membrane be reconciled with evidence that membrane docking requires SNAREs from opposing membranes? SNARE "priming" by NSF may cause transient changes in SNARE conformation that permits them to participate in otherwise unfavorable SNARE–SNARE interactions. Alternatively, NSF-dependent disruption of complexes on each membrane may free SNAREs to form similarly aligned complexes between partners from opposing membranes. Such a zipper-like interaction might play a role in pulling the bilayers close to each other.

Much evidence points to a link between the functions of Rab proteins and NSF/SNAP activity. However, demonstration of a direct link has proved elusive. The first such evidence has emerged from studies of Ypt1, a yeast Rab involved in ER–Golgi transport. The yeast protein Sly1p binds to a tSNARE, Sed5p, which prevents binding of Sed5p to its cognate v-SNAREs, Sec22p and Bet1p. V. Lupashin (Princeton University) showed evidence that Ypt1p promotes formation of the SNARE complex, possibly by binding directly to Sed5p, displacing Sly1p and permitting a Sed5p/v-SNARE interaction to occur (Lupashin and Waters, 1997). In this way the Rab protein would regulate the availability of the t-SNARE.

Most intracellular fusion events are associated with the vesicular or tubular transport of cargo between stable compartments, or homotypic fusion events that contribute to the organization of a compartment. Evidence for a novel variant was presented by P. Luzio (Cambridge University). Using a cell-free system, Luzio found that preexisting lysosomes fuse with late endosomes in an NSF-dependent and Rab-GDI-sensitive manner to form a hybrid organelle that can be separated from both participating organelles by density gradient centrifugation. The hybrid organelles contain endocytosed markers, mannose-6-phosphate receptors, and lysosome-derived enzymes. Evidence that similar hybrid organelles form in vivo was presented.

Membrane Lipids: Integration of Structural and Signaling Functions

The times when the role of membrane lipids in endocytosis seemed strictly structural are over! One-third of the presentations at this conference implicated specific lipids at various stages of the endocytic process. Endocytic membranes and plasma membranes contain significantly more phosphatidylserine (PS), sphingomyelin (SM), glycolipids, and sterols than other organelles, with less phosphatidylcholine (PC). T. Kobayashi (University of Geneva) reported that antibodies from patients suffering from the so-called antiphospholipid syndrome specifically recognize lysobisphosphatidic acid (LBPA). LBPA is highly enriched in the internal membranes of late endosomes of BHK cells, which appear to be involved in sorting/trafficking of the multifunctional receptor IGF2/MPR.

Lipids are distributed asymmetrically across the mammalian plasma membrane bilayer. Whereas PC, SM, and glycolipids are enriched in the exoplasmic leaflet, the aminophospholipids, notably PS, are concentrated on the cytosolic face by the aminophospholipid translocator. E. Farge (Institut Curie, Paris) reported the observation with Dautry-Varsat that insertion of exogenous PS

analogs into the outer leaflet of the plasma membrane of K562 cells resulted in internalization of this PS and an increase in bulk-flow and clathrin-mediated endocytosis, supporting the idea that inward translocation of PS molecules by this ATPase could build up a pressure in the cytosolic leaflet of the plasma membrane that in turn could drive endocytic budding. These data would suggest that lipid translocation across the plasma membrane needs to be carefully regulated. First, the PS translocator appears to be complexed with accessory protein(s). Second, various translocators may move lipids in the opposite direction (Zwaal and Schroit, 1997). G. van Meer (University of Amsterdam) discussed the finding that not only the MDR1 P-glycoprotein but also the multidrug resistance-associated protein MRP1 can translocate various lipids from the cytosolic to the exoplasmic leaflet of the plasma membrane.

Sphingolipids, cholesterol, and GPI-anchored proteins supposedly form domains in the exoplasmic leaflet of the plasma membrane, with caveolae being a subclass of such domains. Parton reported that cholesterol-binding agents disrupt both fibroblast caveolae and T-tubule development in muscle cells, which suggests a role for caveolae-like structures in T-tubule development. Since so many proteins have been assigned to caveolae based on their presence in low-density membranes after detergent extraction at 4°C, it seems sensible to apply additional criteria. S. Vilaro (University of Barcelona) reported identification of p43 as a novel caveolar protein in HEp2 cells by detergent-insolubility and immuno-gold/deep-etch microscopy.

Lipid domains are thought to play a role in the establishment of epithelial surface polarity by being included specifically into the apical pathway from the *trans*-Golgi network (TGN) (Simons and Ikonen, 1997). In order for lipids to play a role in protein sorting, the lipids themselves must be sorted. Most work on epithelial lipid sorting has made use of short-chain lipid analogs of SM and glycolipids, and G. van Meer reported that the apical enrichment of fluorescent glucosylceramide in the original studies on MDCK cells was caused by an MDR-like translocator. For the transcytotic sorting of these fluorescent lipids in HepG2 cells, this is apparently not the case (S. van Ijzendoorn and D. Hoekstra, University of Groningen). Also, inhibitors of MDR1 and MRP1 had no effect on the biosynthetic sorting of radiolabeled lipid analogs, which supports the involvement of lipid domains.

PS on the cytosolic face of the plasma membrane is known to bind a number of cytosolic proteins. V. Gerke (University of Münster) discussed how annexin II can aggregate endocytic vesicles by annexin-typical binding to negatively charged lipids. The process required dimerization of annexin II in a complex with two copies of the protein p11.

Of special interest are the phosphorylated derivatives of phosphatidylinositol (PI) that can be produced locally and may recruit proteins involved in membrane budding to specific membranes at specific times. PH domains with varying binding specificities for phosphoinositides have been identified in a number of these proteins including ARF1, AP2, AP180, dynamin, synaptotagmin (de Camilli et al., 1996), a nucleotide exchange factor for ARF6 (EFA6), and the ARNO family of ARF1 exchange factors (P. Chavrier, Center of Immunology, Marseille). PI-3P is recognized by the Rab5-binding protein EEA1 (Stenmark), and is required for macropinocytosis in fibroblasts (Courtoy), apical macropinocytosis in MDCK cells (Sandvig), early endosome fusion, and late endosome–lysosome fusion in vitro (Luzio). While 5-phosphatases like synaptojanin may inactivate signaling lipids, little is known about the involvement of phospholipases C in terminating phosphoinositide action.

Riezman reported two requirements for lipids in endocytic internalization in yeast. Yeast mutants deficient in ergosterol biosynthesis are defective for endocytosis, but not for secretion. A yeast mutant with a temperature-sensitive defect in serine palmitoyltransferase, the first enzyme in ceramide synthesis, shows a rapid defect in endocytosis upon temperature shift. This rapid loss in endocytic capacity cannot be due to depletion of cell surface sphingolipids. They could show that a signaling function of phytosphingosine or ceramide is involved that is transmitted through a ceramide-activated protein phosphatase.

The Endocytic Pathway in Immunity

Class II major histocompatibility molecules (MHC II) molecules contain two transmembrane polypeptides, α and β, that carry the peptide-binding groove in which antigenic peptides are bound for presentation to T cells. To prevent peptide binding to MHC II in the ER, newly synthesized $\alpha\beta$ complexes associate with a third polypeptide, the invariant chain (Ii), that temporarily occupies the peptide-binding groove. Ii also spans the membrane and its cytoplasmic domain contains information that targets the $\alpha\beta$ complex to the endocytic pathway. H. Geuze (Utrecht University) presented detailed immuno-cryo-EM of human and mouse B lymphoblasts labeled for endocytic markers, Ii, loaded MHC II, and various endogenous markers of endocytic organelles. Their results indicate that $\alpha\beta$Ii complexes first appear in a compartment largely depleted of transferrin receptor and presumed to be just downstream of the early endosome compartment, and that the first peptide-loaded complexes can also be detected in this compartment. Significantly, the organelles containing MHC II and Ii appear to be normal compartments of the endocytic pathway and not compartments specialized for antigen presentation (Kleijmeer et al., 1997).

Three leucine-based sorting signals (two in Ii and one in MHC-II) appear to be required for endosomal targeting of the $\alpha\beta$Ii complex. Using a novel phage display screen, O. Bakke (University of Oslo) found that leucine-based signals (and tyrosine-based signals) can interact with phage-expressed peptides representing sequences from the μ chains of the AP-1 and AP-2 adaptor complexes. Using an assay established to analyze the adaptor recruitment to mannose-6-phosphate receptors located in the TGN, B. Hoflack found that expression of $\alpha\beta$Ii allows recruitment of AP-1 complexes. Substitution of either of two leucine signals in the cytoplasmic domain of Ii reduced binding, and a double substitution abolished association, suggesting that exposure of both signals may be required for optimal AP-1 association and

sorting. Significantly, mutation of Ser9 in Ii also abolished AP-1 binding. The fact that this residue can be phosphorylated may indicate that Ii-mediated sorting of αβ can be regulated by phosphorylation.

The interaction of Ii with AP-1 suggests that transport of αβIi from the TGN occurs in clathrin-coated vesicles. However, expression of the hub domain of clathrin, which inhibits coated vesicle-mediated transport (see above), appears to have no effect on the delivery of newly synthesized αβIi to the endocytic pathway (F. Brodsky) whereas DM, an MHC II-linked protein that facilitates peptide loading onto MHC II, is trapped in the TGN. This suggests that DM and αβIi are sorted to the endocytic pathway by different sets of transport vesicles, one clathrin-dependent (DM) and the other not (αβIi). However, the apparent involvement of AP-1 (see above) in this sorting still has to be reconciled. It will be interesting to see if the newly described AP-3 complex has a role in these sorting/transport events, especially as AP-3 appears to mediate transport independently of clathrin and bind dileucine signals. Significantly, the defect in Chediak-Higashi syndrome may be associated with an inability to sort αβIi and other membrane proteins of late endosomes/lysosomes to their correct destinations (S. Amigorena, Institut Curie, Paris), though how this defect gives rise to the giant lysosomes characteristic of cells from C-H patients remains unclear.

The antigens that provide the peptides for presentation on MHC II molecules can be acquired by multiple endocytic routes. Regardless of the specific pathway used, the most effective means to capture antigens is through the use of specific cell surface receptors of which the immunoglobulin molecules expressed on B lymphocytes and the Fc receptors on macrophages and other antigen-presenting cells (APCs) are among the best characterized. Fc receptors come in multiple forms with different affinities for Ig isoforms. Significantly, the cytoplasmic domains of several FcR family members contain ITAM (or ARAM) motifs that are substrates for Src family tyrosine kinases and, in their phosphorylated forms, binding sites for the non-membrane-associated Zap-related kinase Syk. Amigorena presented evidence that the intracellular trafficking of FcγRIII, and its ability to deliver antigens to the MHC II–loading compartment, may be coupled to the ability of this receptor to recruit Syk. Site-specific mutations in the FcgRIII γ chain that did not affect internalization, inhibit transport to late endosomes/lysosomes, inhibit degradation of internalized ligand, and inhibit Syk kinase activation.

For presentation to T cells the antigen-loaded αβ complex must be transported to the cell surface. Geuze and colleagues have suggested that MIICs may fuse directly with the plasma membrane in a process resembling the shedding of transferrin receptor–containing membranes from reticulocytes (Raposo et al., 1996). However, it is unclear how MHC II molecules reach the cell surface from other locations in the endocytic pathway, in particular the CIIV compartment (see below). Watts (University of Dundee) demonstrated that for one particular T cell epitope αβ transport to the cell surface must occur from a compartment beyond the itinerary of the transferrin receptor. Using transferrin-HRP, they demonstrated that ablation of the sorting and recycling endosome compartments normally accessed by transferrin does not prevent transport to the cell surface of αβ complexes capable of presenting antigen to T cells. However, this route does appear to be sensitive to brefeldin A, suggesting that coated vesicles of some sort may be involved in transport.

Data presented at this meeting provided some explanation for the apparent differences in previous experiments addressing the location of MHC II–peptide loading and suggested a mechanism through which MHC II can be differentially distributed through endocytic organelles. As recently reported, MHC II in immature dendritic cells is located primarily in late endosomes/lysosomes equivalent to MIICs. In contrast, in intermediate-stage cells MHC II complexes are found in endosomal organelles with the characteristics of CIIVs, and in mature cells most MHC II is at the cell surface. Significantly, antigens acquired by early-stage cells can be presented efficiently by mature cells. Mellman (Yale University Medical School) suggested that the timing of Ii removal from the αβ complexes may contribute to these different distributions. Ii is removed through a series of proteolytic cleavages. Cathepsin S specifically removes a fragment containing the transmembrane and cytoplasmic domain of Ii (p10K) and so removes the trafficking signals from αβ. It appears that in immature dendritic cells cleavage of p10K is slow, resulting in αβIi being transported to MIICs from where cycling to the cell surface is slow. In mature cells p10K is removed earlier with the result that αβ can recycle early from CIIVs. The different proteolytic activities in immature and mature cells appears not to be a consequence of differences in protease expression, but possibly in the levels of an endogenous inhibitor of cathepsin S activity. Thus, differences in the association of escort proteins with αβ, mediated through regulation of the proteolytic environment within the endocytic pathway, controls the cellular distribution of MHC II.

The Endocytic Pathway in Disease

While many toxins or pathogens enter the endocytic pathway and are effectively neutralized by the immune response, others are able to circumvent this process and to exploit the endocytic system to their own benefit. For example, the VacA toxin produced by Helicobacter pylori is responsible for the development of large vacuoles derived from the late endocytic pathway. E. Papini (University of Padova) demonstrated that these vacuoles are Rab7-containing late endosomes and that vacuolation is dependent on functional Rab7. Since Rab7 regulates fusion between late endosomes in vitro, VacA may induce vacuolation by increasing the extent of homotypic fusion between late endosomes or perhaps by altering membrane flux through this compartment. The effect on the pH of this compartment probably affects its sorting properties.

Aeromonas hydrophila produces a pore-forming toxin (aerolysin) that also induces vacuolation in infected cells (G. van der Gout, University of Geneva). However, the specific compartment affected in this case is the ER. Vacuolation is microtubule-dependent, and is accompanied by release of the vesicle coat protein β COP into the cytosol and a block in ER–Golgi transport. This

striking effect on cell function is likely to be indirect because the toxin itself forms pores only in the plasma membrane. Most likely, efflux from the cell of K^+ or other ions plays a role. van der Goot also presented evidence demonstrating how aerolysin-containing pores are formed. Aerolysin is synthesized as a hydrophilic protoxin dimer that binds to an 80 kDa GPI-anchored protein found in cholesterol-enriched domains of the plasma membrane. Such localized enrichment of pro-aerolysin is probably necessary for pore formation, since pores contain hexamers of the mature toxin. Toxin activation is effected by a host cell protease, possibly furin, and leads to a marked increase in hydrophobicity that could drive membrane insertion.

Mycobacteria persist in their host cell within vacuoles that are integral to the recycling endosomal system (D. Russell, Washington University) but which have been modified to assist the pathogen (Sturgill-Koszycki et al., 1996). In particular, the vacuolar membranes are depleted of vacuolar ATPase. Hence, they show limited acidification and restricted hydrolytic activity despite possessing lysosomal proteases. Even in established infections, they remain dynamic compartments accessible to several plasma membrane–derived constituents. Thus, they provide ideal sites for mycobacterial survival. Mycobacterial infection also provides an excellent example of how host–pathogen interactions determine the success of pathogen infection and/or persistence, a theme of several presentations. Although mycobacteria persist in quiescent macrophages, activation by cytokines from a productive cellular immune response stimulate the cells to kill their resident pathogens. This is associated with coalescence of the mycobacterial vacuoles to form more acidified compartments that contain multiple bacilli. Mycobacteria have developed several strategies to avoid this outcome. Most notably, live bacilli induce sustained release of IL-6 from infected macrophages. IL-6 blocks the ability of T cells to respond to appropriate stimuli. This could render the centers of infection foci, such as granulomas, anergic and thus avoid release of macrophage-activating cytokines.

Using Brucella abortus as a model, J.-P. Gorvel (Center of Immunology, Marseille) also showed how macrophage activation can affect the fate of pathogens. NF-IL6 is implicated in the expression of a number of activated macrophage-specific cytokines. The role of NF-IL6 in bacterial killing was investigated using activated macrophages harvested from NF-IL6 $(-/-)$ or $(+/-)$ mice. A strain of B. abortus that is killed effectively by macrophages from wild-type mice persisted in activated macrophages from NF-IL6 $(-/-)$ mice. Bacterial survival was associated with reduced endocytic uptake compared with heterozygous control cells. However, the most pronounced feature was an inability of bacterial phagosomes to interact with the host endocytic pathway. Gorvel provided evidence that G-CSF expression, which was lowered in NF-IL6 $(-/-)$ macrophages, was necessary for increased endocytosis, phagosome-early endosome fusion and bactericidal activity. The underlying mechanism for G-CSF activity here is not known. It will be interesting to see if G-CSF affects fusion between bacteria-free endocytic compartments, or if it modulates the activity of any of the fusion-promoting proteins discussed above.

A more unusual exploitation of the endocytic system is that shown by the lentiviruses. M. Marsh (MRC LMCB, London) demonstrated that in the case of simian immunodeficiency virus (SIV), endocytosis signal(s) within the cytoplasmic domain of the envelope protein (Env) may be important in determining pathogenesis. Analysis of the cytoplasmic domain of Env, together with studies using chimeras, suggests that a sequence, similar to the lysosomal sorting motif found within LAMP-1, is critical for the function of one sorting signal. However, at least one other signal is also present. Endocytosis of Env seems at first glance an unlikely virulency factor. However, Marsh argued that endocytosis of newly synthesized envelope proteins in infected cells might drive budding from an internal membrane and thus enhance the ability of virus to evade the host immune system.

Studies of HIV infection provide another example of how host responses can influence the success of pathogens. Entry of HIV/SIV requires both the CD4 receptor and a chemokine receptor on the host cell. Unlike CD4, surface expression of CCR5 and CXCR4 is down-regulated by their ligands (RANTES and SDF-1, respectively) and thus provides a potential mechanism for host systems to prevent viral entry. Marsh showed that endocytosis of the CXCR4 is increased in response to phorbol esters or SDF-1 binding. Internalization is via the classical coated pit pathway and requires a determinant(s) within the COOH-terminal cytoplasmic domain of the receptor. In addition, chemokines could indeed protect cells from SIV infection, but not if they expressed truncated forms of CXCR4 lacking this domain. J. L. Carpentier (University of Geneva) studied the role of SIV and HIV early protein Nef in down-regulation of CD4. He showed that Nef protein increased the association of CD4 with clathrin-coated pits by inducing coated pit formation. CD4 has a dileucine motif that is required for its down-regulation. SIV and HIV-2 Nef interacts with $\mu 2$ by the yeast two-hybrid system. In addition, a direct replacement of the cytoplasmic tail of CD4 with Nef allowed CD4 internalization. It will be interesting to see whether this function as a connector between CD4 and AP-2 is required for efficient endocytosis of all proteins with dileucine motifs or whether some of these can interact directly with AP-2.

Conclusions and Perspectives

One of the outstanding outcomes of this meeting was the identification of a large number of molecules that are required for endocytic vesicle formation. The need for a large number of proteins at this step is not yet clear, but some possible explanations can already be put forward. First, several of the newly identified proteins have putative interactions with the actin cytoskeleton. If the actin cytoskeleton plays a direct role in endocytic internalization, this could help explain the complexity of the process. Second, several of the newly identified proteins have some relation (binding domains or metabolic activities) to lipids, in particular signaling lipids, such as phosphorylated inositides or ceramides. Therefore, the observed complexity could reflect a need to integrate endocytosis, which includes receptor down-regulation, with signal transduction. One of the challenges of the future is to understand the connections

between endocytosis, the actin cytoskeleton, and signal transduction in greater detail. Some of the potential molecules that could connect the two pathways functionally were described at this meeting and their similarity in structure and interaction patterns suggest that many aspects of endocytosis may be conserved from yeast to vertebrates. This should allow a more comprehensive use of genetics to help understand and test our hypotheses. Obviously, the new players in the endocytic pathway give us leads to the discovery of additional components. New assays will probably be required to understand the functions of these molecules in the process of endocytosis.

Progress has also been made in our understanding of fusion events along the endocytic pathway. Again, several new components have been implicated in this pathway since the last meeting, and our models of how membrane fusion occurs are still evolving. Fusion events along the endocytic pathway remain one of the most productive systems to study biological membrane fusion in general.

Finally, our increased understanding of the endocytic pathway had helped us to understand various problems in health and disease. Studies of endocytosis have helped us to understand how antigens are presented, but the converse is also true. Antigen presentation seems to use a rather normal endocytic system, but this pathway has to be exploited to suit particular needs in antigen presentation. In particular, interesting developmental changes in antigen-presenting cells were presented at this meeting suggesting ways in which we may be able to understand other aspects of the immune system at the molecular and cellular level. A similar situation exists for understanding diseases. The endocytic system plays an important role in resistance to disease and our understanding of how certain pathogens can override this resistance may lead to novel methods in disease control.

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